ATTACHMENT B

(Dudek and Garcia (Cytoskeletal regulation of pulmonary vascular permeability, *J Appl Physiol* 91:1487-1500, 2001)

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invited review

# Cytoskeletal regulation of pulmonary vascular permeability

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**Dudek, Steven M, and Joe G. N. Garcia.** Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 91: 1487–1500, 2001.—The endothelial cell (EC) lining of the pulmonary vasculature forms a semipermeable barrier between the blood and the interstitium of the lung. Disruption of this barrier occurs during inflammatory disease states such as acute lung injury and acute respiratory distress syndrome and results in the movement of fluid and macromolecules into the interstitium and pulmonary air spaces. These processes significantly contribute to the high morbidity and mortality of patients afflicted with acute lung injury. The critical importance of pulmonary vascular barrier function is shown by the balance between competing EC contractile forces, which generate centripetal tension, and adhesive cell-cell and cell-matrix tethering forces, which regulate cell shape. Both competing forces in this model are intimately linked through the endothelial cytoskeleton, a complex network of actin microfilaments, microtubules, and intermediate filaments, which combine to regulate shape change and transduce signals within and between EC. A key EC contractile event in several models of agonist-induced barrier dysfunction is the phosphorylation of regulatory myosin light chains catalyzed by $Ca^{2+}$/calmodulin-dependent myosin light chain kinase and/or through the activity of the Rho/Rho kinase pathway. Intercellular contacts along the endothelial monolayer consist primarily of two types of complexes (adherens junctions and tight junctions), which link to the actin cytoskeleton to provide both mechanical stability and transduction of extracellular signals into the cell. Focal adhesions provide additional adhesive forces in barrier regulation by forming a critical bridge for bidirectional signal transduction between the actin cytoskeleton and the cell-matrix interface. Increasingly, the effects of mechanical forces such as shear stress and ventilator-induced stretch on EC barrier function are being recognized. The critical role of the endothelial cytoskeleton in integrating these multiple aspects of pulmonary vascular permeability provides a fertile area for the development of clinically important barrier-modulating therapies.

cytoskeleton; endothelium; actomyosin contraction; actin-binding proteins; acute lung injury

## PULMONARY VASCULAR BARRIER REGULATION: OVERVIEW

Despite recent therapeutic advances, inflammatory pulmonary conditions such as acute lung injury, acute respiratory distress syndrome, and sepsis continue to result in high rates of patient morbidity and mortality (3). Centrally involved in the pathogenesis of these processes and now recognized as a cardinal feature of inflammation, increased vascular permeability contributes to the profound pathophysiological derangements observed in these disorders. Because of the enormous surface area of the pulmonary vasculature, the pulmonary endothelium, which functions as a semipermeable cellular barrier between the vascular compartment and the interstitium, is particularly sensitive to

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the dynamic features of barrier regulation. Endothelial barrier properties are not uniform throughout the pulmonary vasculature, with greater macromolecule diffusion in postcapillary venules compared with pulmonary arterioles in whole lung models (91, 96, 118), whereas cultured microvascular endothelial cells (ECs) exhibit tenfold higher barrier properties than macrovascular EC as measured by electrical resistance across monolayers (13). Although the precise mechanisms that regulate this variability in segmental barrier function are unknown, barrier regulatory components such as $Ca^{2+}$ signaling pathways and differences in content and regulation of barrier protective cAMP are likely involved (21, 80, 135).

The integrity of the pulmonary EC monolayer is a critical requirement for preservation of pulmonary function, with two general pathways described for the movement of fluid, macromolecules, and leukocytes into the interstitium and subsequently the alveolar air spaces. The transcellular pathway utilizes a tyrosine kinase-dependent, gp60-mediated transcytotic albumin route, whose regulation and function are unclear but which may serve to uncouple protein and fluid permeability (103, 127, 143). However, there is general consensus that the primary mode of fluid and transendothelial leukocyte trafficking occurs by the paracellular pathway (Fig. 1), whose essential role in endothelial permeability has been well supported by an impressive body of research, including electron microscopy studies (69, 102), which demonstrate the formation of paracellular gaps at sites of active inflammation within the vasculature.

Mechanistic approaches designed to understand EC paracellular gap formation and barrier function have revealed the complexity of these processes; however, several valuable paradigms have been developed. One useful model describes paracellular gap formation as regulated by the balance of competing contractile forces, which generate centripetal tension, and adhesive cell-cell and cell-matrix tethering forces, which together regulate cell shape changes (53). As outlined in Fig. 2, both competing forces in this model are intimately linked to the actin-based endothelial cytoskeleton by a variety of actin-binding proteins that are critical to both tensile force generation as well as to linkage of the actin cytoskeleton to adhesive membrane components. In this review, we will highlight the essential role of the EC cytoskeleton in linking these elements to the modulation of vascular permeability within the pulmonary circulation.

## EC CYTOSKELETON

The cytoskeleton is composed of three primary elements: actin microfilaments, intermediate filaments, and microtubules. Actin filaments are of critical importance to EC permeability, as demonstrated by the findings that cytochalasin D, a well-described disrupter of the actin cytoskeleton, increases EC permeability in cultured cells (129), whereas phallacidin, an actin stabilizer, prevents agonist-mediated barrier dysfunction (117). The actin microfilament system is focally linked to multiple membrane adhesive proteins such as cadherin molecules, glycocalyx components, functional intercellular proteins of the zona occludens (ZO) and zona adherens, and focal adhesion complex proteins (Fig. 2). Actin structures are also intimately involved in EC tensile force generation. ECs contain an abundance of the molecular machinery necessary to generate tension via an actomyosin motor, actin and myosin represent ~16% of total endothelial protein (159), and focally distributed changes in tension and relaxation

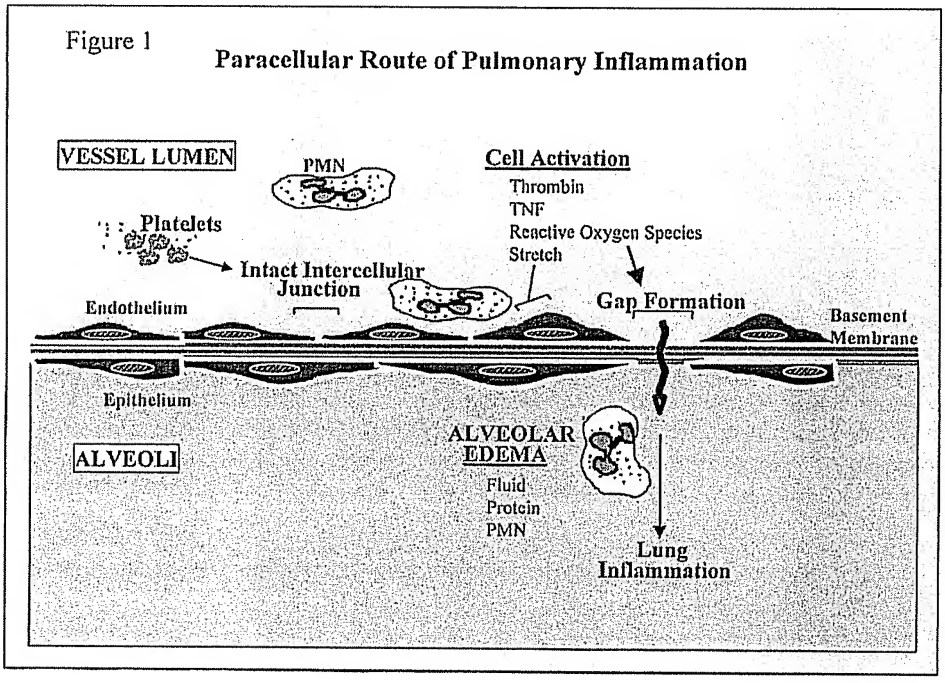


Fig. 1. Paracellular route of pulmonary inflammation. Under basal conditions, endothelial cells (ECs) of the pulmonary vasculature form a semipermeable barrier that restricts the flow of luminal contents into the alveolar air spaces. The important roles of flow and platelets/platelet-derived phospholipids in maintaining these intact intercellular junctions are becoming increasingly recognized (52, 147, 166, 167). During inflammation, the endothelium is activated by biophysical alterations (stretch or increased shear) or by stimuli such as thrombin, tumor necrosis factor (TNF), and/or reactive oxygen species to form paracellular gaps. In concert with a break in the EC barrier, fluid, proteins, and polymorphonuclear neutrophils (PMNs) flow into the alveoli to produce pulmonary edema via this paracellular route.

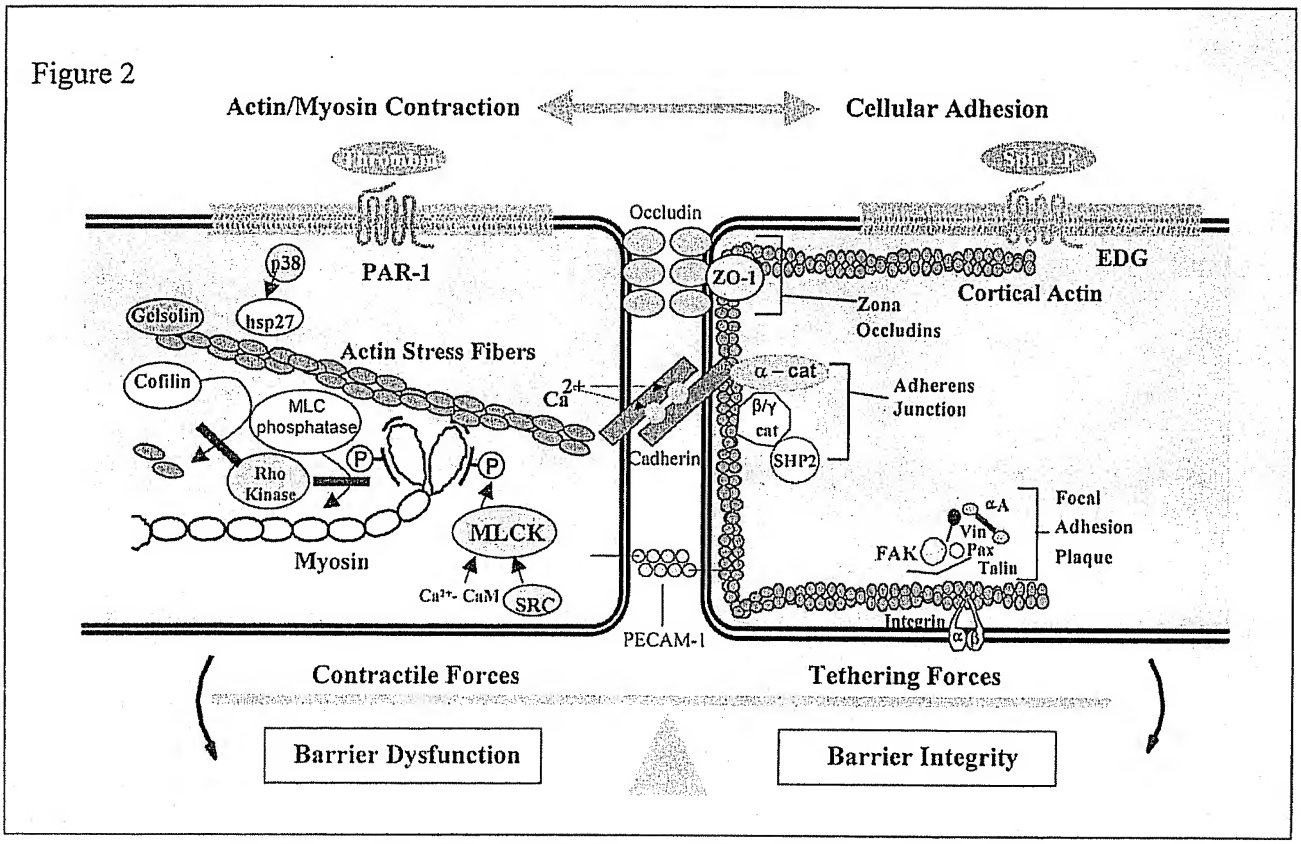


Fig. 2. Actomyosin contractile elements and cellular adhesive forces regulate endothelial paracellular gap formation. In this working model of EC barrier regulation, under basal conditions, a balance exists between actomyosin contractile and cellular adhesive forces. When contractile forces predominate, as depicted in the thrombin-stimulated model (*left*), ECs pull apart to form paracellular gaps, favoring barrier disruption. *Left*: schematic representation of the major components involved in regulating endothelial actomyosin contraction. Thrombin binding to its receptor increases intracellular $Ca^{2+}$, which via $Ca^{2+}$/calmodulin (CaM) interaction activates myosin light chain kinase (MLCK) to phosphorylate (P) myosin light chains (MLCs), leading to increased actomyosin interaction, force development, and subsequent contraction. Also depicted are other representative regulatory proteins whose activity either increases (green background) or decreases (white background) EC tension. Tyrosine phosphorylation of EC MLCK catalyzed by $pp60^{src}$ (SRC) increases MLCK kinase activity, whereas Rho kinase augments EC contraction by inhibiting both MLC phosphatase and the actin-depolymerizing protein, cofilin. Inhibition of heat shock protein (HSP) 27 activity by p38 mitogen-activated protein kinase (MAPK)-catalyzed phosphorylation induces actin stress fiber formation, and the actin capping/severing protein gelsolin is likewise involved in stress fiber-dependent contraction. *Right*: cell-cell and cell-matrix contacts that link ECs into a functional barrier between the vasculature and the airways. When these tethering forces predominate, as in the sphingosine 1-phosphate (Sph-1-*P*) model, a thick cortical actin ring is observed, whereas ECs maintain tight connections with each other and the underlying matrix to tilt the balance toward increased barrier integrity (167). Cell-cell connections include tight junctions composed of transmembrane occludin proteins linked to the EC actin cytoskeleton by the zona occludins family (ZO-1), adherens junctions mediated by $Ca^{2+}$-dependent association of cadherin proteins in turn linked to the α-, β-, γ-catenin (cat) complex, and platelet endothelial cell adhesion molecule-1 (PECAM-1)-associated junctions. An example of the complicated regulation of these adhesive sites is provided by the tyrosine phosphatase SHP2, which appears to help stabilize adherens junctions by decreasing tyrosine phosphorylation of catenins. Cell-matrix tethering is maintained by focal adhesion plaques composed of α- and β-integrin transmembrane proteins linked to the actin cytoskeleton by a complex of proteins, including talin, paxillin (Pax), vinculin (Vin), α-actinin (αA), and focal adhesion kinase (FAK). PAR-1, protein-activated receptor-1; EDG, endothelial differentiation gene.

can be accomplished by regulation of the level of myosin light chain (MLC) phosphorylation and actin stress fiber formation. There is excellent association between the development of transcellular actin cables, stress fibers, increased MLC phosphorylation, and enhanced tension development, with a key regulator of the EC contractile apparatus being the $Ca^{2+}$/calmodulin (CaM)-dependent MLC kinase (EC MLCK) (an enzyme discussed in detail below).

The actin cytoskeleton is a dynamic structure that undergoes rearrangement under the control of various actin binding, capping, nucleating, and severing proteins, which are intimately involved in regulating the contractile status of cells (Table 1). For example, the actin-depolymerizing activity of cofilin is inhibited by Rho-GTPase pathway activation during stress fiber formation (101). In addition, reduction in either expression or activity of the abundant actin-severing

Table 1. *Representative actin-binding proteins with potential endothelial barrier regulatory function*

| | | Molecular Mass, kDa | Splice Variants/ Isoforms |
|---|---|---|---|
| | *Cross-linking/bundling proteins* | | |
| Spectrin | Cross-links F actin at periphery and stimulates myosin II ATPase (138) | 260/240 | + |
| α-Actinin | Links actin cytoskeleton to focal adhesions (36) Displacement from actin stress fibers disrupts the microfilaments (114) | 100 | |
| Fimbrin | Links actin cytoskeleton to vimentin network at cell adhesion sites (26) | 68 | + |
| Cortactin*† | Phosphorylation by $p60^{src}$ reduces actin bundling activity (74) | 80 | + |
| | *Polymerization/depolymerization proteins* | | |
| Cofilin | Rho pathway inhibits depolymerization activity during stress fiber formation (101) | 20 | |
| hsp27* | Phosphorylation by p38/MAPKAP induces stress fiber formation (121, 126) | 27 | |
| VASP* | Stimulates actin nucleation and polymerization at focal adhesions (76) | 45 | |
| Arp2/3 | Protein complex produces branching actin network at cell periphery through interaction with Wiskott-Aldrich syndrome protein (WASP) and cortactin (100, 147) | 215 | |
| Profilin | Overexpression inhibits actin stress fiber formation (105) | 14 | |
| | *Capping/severing proteins* | | |
| Gelsolin | Inhibition of activity decreases stress fiber-dependent contraction (4) | 83 | |
| | *Miscellaneous actin binding proteins* | | |
| MLCK*† | Activation produces stress fibers, cellular contraction, EC permeability (49, 141, 161) | 210 | + |
| Filamin*‡ | Phosphorylation by CaMKII permits reorganization of cortical actin (14) | 280 | |
| Caldesmon* | Facilitates actomyosin interaction (66, 134) | 77 | + |
| Vinculin | Binds actin and catenin at junctional sites (125, 156) | 130 | + |

Representative actin-binding proteins are grouped by category of actin interaction (cross-linking, bundling, capping, and so forth) and accompanied by a brief description of their known or postulated role in endothelial cell (EC) barrier regulation. The molecular masses for the primary isoforms or variants in EC are listed in kDa for each protein. Additional complexity in the system is provided by multiple isoforms or splice variants (+) for several of the actin-binding proteins. CaMKII, calmodulin kinase II. *Activity and/or function regulated by serine/threonine phosphorylation. †Activity and/or function regulated by tyrosine phosphorylation.

protein gelsolin significantly decreases stress fiber-dependent contraction in cultured cells (4). Another actin-binding protein involved in cellular contraction is the 27-kDa heat shock protein (HSP27), whose actin-binding properties are altered by phosphorylation through a p38 mitogen-activated protein kinase (MAPK)-driven pathway. Reduction of HSP27-induced inhibition of actin polymerization alone can produce stress fiber formation (121, 126). Undoubtedly, important roles for additional F-actin binding proteins in regulating cell contraction, potentially in a splice-variant-specific manner (12), will continue to be elucidated.

The roles of microtubules and intermediate filaments in EC barrier regulation are much less defined. Microtubules are polymers of α- and β-tubulins that form a lattice network of rigid hollow rods spanning the cell in a polarized fashion from the nucleus to the periphery while undergoing frequent assembly and disassembly (153). Historically viewed as separate and distinct cytoskeletal systems, microtubules and actin filaments are now known to interact functionally during dynamic cellular processes (64, 87). Microtubule disruption with agents such as nocodazole or vinblastine induces rapid assembly of actin filaments and focal adhesions (9, 28, 149), isometric cellular contraction (28), which correlates with the level of MLC phosphorylation (88), increased permeability across EC monolayers (149, 155), and increased transendothelial leukocyte migration (85), whereas microtubule stabilization with paclitaxel attenuates these effects. The mechanisms involved in these effects are poorly understood but are likely to be mediated through interaction with actin filaments, suggesting significant microfilament-microtubule cross talk and an intriguing role for the microtubule cytoskeleton in EC barrier regulation.

Intermediate filaments represent the third major element involved in EC cytoskeletal structure. Although they exhibit much greater diversity than the highly conserved components of either actin microfilaments or microtubules, intermediate filament proteins share a common dimer structure containing two parallel α-helices, which combine to form apolar fibrils that associate with an array of intermediate filament-binding proteins while connecting to the nuclear envelope, peripheral cell junctions, and other cytoskeletal components (23, 46). Intermediate filament proteins are expressed in a highly cell-specific manner, with vimentin representing the primary protein found in EC and other cells of mesenchymal origin (39). Vimentin phosphorylation occurs rapidly in thrombin- or phorbol-stimulated endothelium (134); however, the role of vimentin in EC structure and resultant barrier function remains unclear. Early work utilizing an ethchlorvynol-induced model of EC permeability failed to demonstrate any effects on intermediate filament structure (160). Although a more recent report describes dramatic alteration of actin and microtubule filaments in cultured cells after peptide-induced vimentin disassembly (63), fibroblasts derived from vimentin knockout mice displayed normal actin and microtubule architecture, whereas the animals themselves developed normally without any obvious phenotypic abnormalities (24). These data suggest that potential roles for intermediate filaments in EC cytoskeletal structure,

and more specifically barrier function, are likely to be subtle and subject to compensation by biologic redundancy.

## REGULATION OF VASCULAR PERMEABILITY BY THE EC CONTRACTILE APPARATUS

Multiple studies have demonstrated a critical role for activation of the contractile apparatus in specific models of agonist-induced EC barrier dysfunction. A well-studied model is that evoked by thrombin, a central regulatory molecule in the coagulation cascade. The dual finding of microthrombi in the pulmonary microvasculature of patients expiring from acute lung injury and recent success of anticoagulant strategies at microcirculatory sites of inflammation (8) illustrates the relevance of this model to the study of acute lung injury and barrier regulation. Thrombin increases pulmonary lymph flow in awake sheep and also increases lung weight gain and reduces the sigma reflective coefficient in the isolated perfused lung, consistent with enhanced permeability (71, 98, 99). In vitro, thrombin induces a profound increase in EC albumin permeability and a reduction in electrical resistance reflective of a loss of barrier integrity through rapid actin cytoskeletal rearrangement and force generation dependent on actomyosin interaction, effects confirmed by fluorescent microscopy and biophysical measurements (49, 50, 89, 125). A key EC contractile event in several models of agonist-induced barrier dysfunction is the phosphorylation of regulatory MLCs catalyzed by $Ca^{2+}$/CaM-dependent MLCK, which is sufficient to produce EC contraction and barrier dysfunction (141, 161). The inflammatory agonists thrombin and histamine both produce rapid increases in MLC phosphorylation, actomyosin interaction, and EC permeability, which can be significantly attenuated by treatment with MLCK inhibitors (50, 148). In addition, MLCK inhibition prevents transforming growth factor-$\beta_1$-stimulated EC permeability (75) and abolishes barrier dysfunction in both rat lung models of ischemia-reperfusion injury (84) and ventilator-induced lung permeability (108). The EC contractile apparatus is activated by polymorphonuclear neutrophil (PMN) adherence and diapedesis with increased MLC phosphorylation, whereas reduction in EC MLCK activity significantly attenuates leukocyte migration (55, 70, 122).

The regulation of the MLCK isoform in the endothelium is complex and differs significantly from smooth muscle MLCK regulation. The only MLCK isoform expressed in ECs is a 1,914-amino acid high-molecular-mass (214 kDa) protein derived from a single gene on chromosome 3 in humans, which also encodes the smaller (130–150 kDa) smooth muscle MLCK isoform (51, 94, 151). EC MLCK shares essentially identical catalytic and CaM regulatory motifs with smooth muscle MLCK (Fig. 3) but in addition includes a unique 922-amino acid $NH_2$ terminus containing multiple sites for protein-protein interactions as well as sites for $p60^{src}$-catalyzed tyrosine phosphorylation, which regulate enzyme activity (12, 51). Protein tyrosine phosphorylation status appears to play an important role in regulation of EC permeability, as demonstrated by the modest enhancement of barrier function with the nonspecific tyrosine kinase inhibitor genistein (17). More specifically, $p60^{src}$-induced tyrosine phosphorylation is critical for diperoxovanadate-induced EC barrier dysfunction through increased contraction and altered focal contacts (54, 131). EC MLCK may provide the link between tyrosine phosphorylation events and permeability changes because tyrosine phosphorylation of EC MLCK evokes significant increases in MLCK activity, EC contraction, and subsequent EC barrier dysfunc-

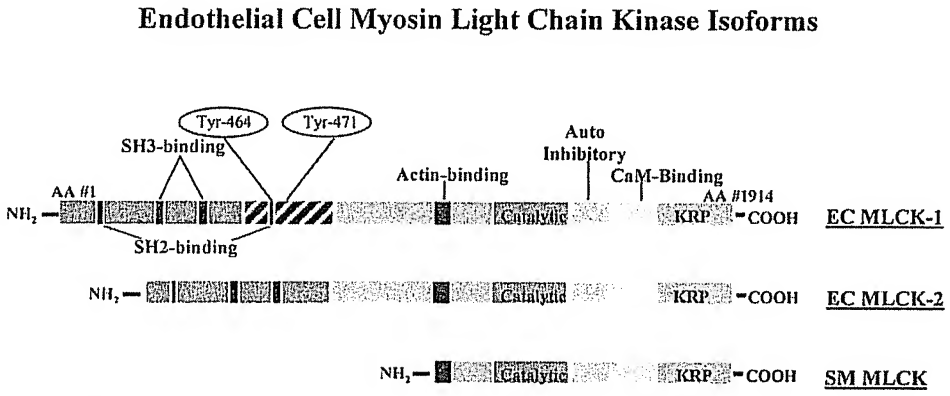


Fig. 3. Representation of isoform/splice-variant-specific contractile regulation: EC MLCK. ECs express a 1914-amino acid, high-molecular-mass isoform of MLCK (EC MLCK 1), which is derived from the same gene on human chromosome 3 as the well-described smooth muscle (SM) isoform. EC MLCK 1 shares with SM MLCK essentially identical actin-binding, catalytic, inhibitory, CaM-binding, and KRP domains in the COOH-terminal half of the protein. However, EC MLCK 1 also contains a unique 922-amino acid $NH_2$-terminal section containing multiple sites for protein-protein interaction (SH2- and SH3-binding domains) as well as potential regulatory phosphorylation sites (68–71). For example, tyrosine (Tyr) residues 464 and 471 have recently been identified as $p60^{src}$ phosphorylation sites that upregulate EC MLCK 1 kinase activity (72). The endothelium also expresses several splice variants of EC MLCK 1; the example shown here is EC MLCK 2, which is identical to isoform 1 except for a single exon deletion encoding 69 amino acids, including the critical tyrosine residues for $p60^{src}$ phosphorylation, and thus is refractory to $p60^{src}$-mediated contractile regulation. KRP, kinase-related protein.

tion while promoting the development of a contractile complex containing EC MLCK, actin, myosin, CaM, $p60^{src}$, and the actin-binding protein cortactin (56, 58, 150).

Recently, the critical importance of the small GTPase Rho in regulation of the contractile apparatus has been demonstrated in several models of agonist-induced EC barrier dysfunction. The Rho family of small GTPases is involved in signal transduction linking extracellular stimuli to dynamic actin cytoskeletal rearrangement (137), and activation of Rho specifically produces stress fiber formation in cultured cells (119). Through its downstream effector, Rho kinase, Rho activation leads to phosphorylation of the myosin binding subunit of MLC phosphatase (PP1), thereby inhibiting its phosphatase activity and resulting in increased MLC phosphorylation, actomyosin interaction, stress fiber formation, and subsequent EC barrier dysfunction (Fig. 4) (2, 38, 86).

The relative contributions of the EC MLCK and Rho pathways in regulating EC permeability are not well understood: inhibition of either MLCK activity (50) or Rho activation (16, 18) attenuates thrombin-induced EC barrier dysfunction. A recent report suggests that Rho/Rho kinase and MLCK may differentially regulate MLC phosphorylation according to spatial localization within cultured cells (144). Additional complexity in the system is provided by the contribution of the p21-activated kinase (PAK) family, downstream effectors of the small GTPases Rac and Cdc42. Isoforms PAK1 and PAK2 have both been shown to phosphorylate smooth

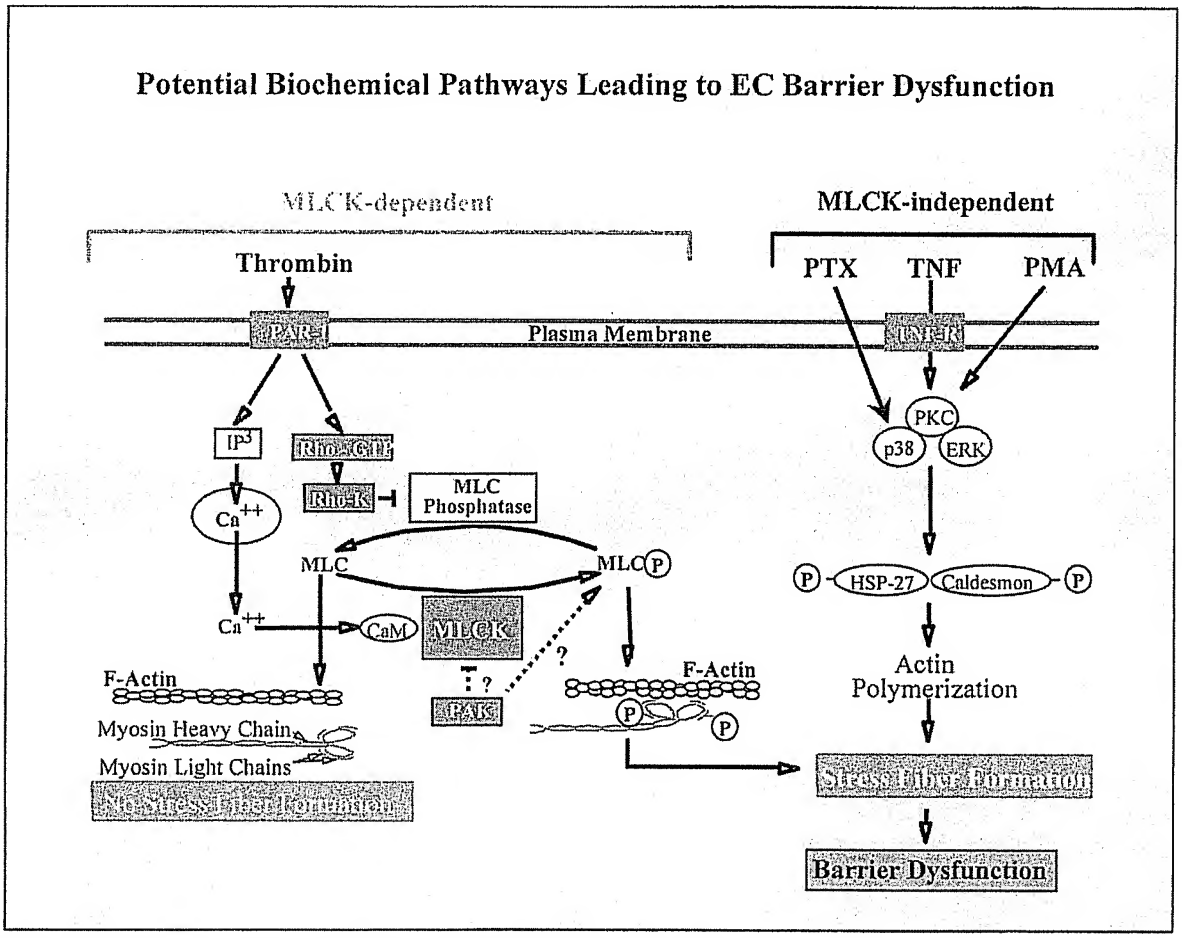


Fig. 4. MLCK-dependent and MLCK-independent pathways are involved in EC barrier dysfunction. MLCK-dependent barrier disruption is represented by the thrombin model outlined at *left*. Thrombin binding to its receptor results in inositol trisphosphate ($IP_3$) production and a subsequent increase in intracellular $Ca^{2+}$, which via $Ca^{2+}$/CaM interaction activates MLCK to phosphorylate MLCs, leading to increased actomyosin interaction and subsequent contraction. Thrombin also increases MLC phosphorylation through Rho/Rho kinase pathway inhibition of MLC phosphatase activity. Examples of MLCK-independent barrier disruption are depicted at *right*. Pertussis toxin (PTX) activates p38 MAPK via an unknown mechanism, resulting in phosphorylation of HSP27, whose inhibitory effect on actin polymerization is thereby attenuated, allowing stress fiber formation to occur. Phorbol myristate acetate (PMA) induces a protein kinase C (PKC)-dependent increase in bovine pulmonary EC permeability without significantly increasing MLC phosphorylation, possibly through extracellular signal-regulated kinase (ERK)-catalyzed caldesmon phosphorylation and subsequent alteration of actomyosin cross bridging. Likewise, the cytokine TNF-α through its receptor (TNF-R) appears to mediate cytoskeletal rearrangement and eventual barrier dysfunction through a PKC-dependent pathway. Although TNF-α produces an increase in MLC phosphorylation, this effect does not appear to contribute to the subsequent increase in EC permeability.

muscle MLCK and decrease MLCK activity in cultured cells (61, 123), but whether PAK regulates the high-molecular-mass MLCK present in endothelium in this fashion is not clear. Conversely, PAK2 can directly phosphorylate MLC to produce EC contraction (163). A complex interplay exists among these processes in regulating MLC phosphorylation status, cell tension, and subsequent EC permeability. Continued exploration of these pathways should provide additional important insights into the regulation of EC barrier function.

Despite the clear contribution of MLCK/Rho kinase-driven increases in MLC phosphorylation to tension development and increased vascular permeability, MLCK-independent pathways are also involved in the regulation of cellular contraction (Fig. 4). Protein kinase C (PKC)-mediated pathways exert a prominent effect on barrier regulation in a time- and species-specific manner. For example, phorbol myristate acetate induces a PKC-dependent increase in bovine pulmonary EC permeability without significantly increasing MLC phosphorylation and without inducing formation of actin stress fibers, whereas PKC activation in human umbilical vein ECs does not have this barrier-disrupting effect (19, 50, 134), likely reflecting differences in PKC isotype-specific expression in the two species. PKC-mediated increases in bovine EC permeability may occur through phosphorylation of caldesmon, an actin-, myosin-, and CaM-binding protein present in smooth muscle actomyosin cross bridges as a 145-kDa protein and in EC as a 77-kDa protein (134). The phosphorylation of caldesmon is known to alter smooth muscle cross-bridge activity (93). Caldesmon distributes along stress fibers and is phosphorylated in EC after thrombin and phorbol myristate acetate challenge (134). Caldesmon-mediated regulation of actomyosin ATPase in smooth muscle is also modified by the actin cross-linking protein filamin and gelsolin (Table 1) (66). Although filamin participates directly in barrier regulation via CaM kinase II activation (14), its effects on actin cytoskeletal rearrangement are regulated through Rho family GTPases (6, 106), thereby providing another link with a known modulator of EC barrier function. Tumor necrosis factor-α (TNF-α) slowly induces barrier disruption in cultured EC, which is independent of MLCK activity (115). Finally, p38 kinase activation has also been linked to contractile regulation in smooth muscle (90), EC migration (97, 121), and lipopolysaccharide-induced EC permeability (164). The mechanism through which p38 exerts these effects is unclear but may involve the actin binding protein HSP27 (162), a known p38 MAPK target whose actin polymerization-inhibiting activity dramatically decreases after phosphorylation (7, 45) in association with stress fiber development (121, 126).

## ADHESIVE PROTEIN-CYTOSKELETON LINKAGES

Cell-cell and cell-matrix adhesions are essential for barrier maintenance and restoration and exist in dynamic equilibrium with EC contractile forces. Intercellular contacts along the endothelial monolayer consist primarily of two types of complexes, adherens junctions and tight junctions, which link to the actin cytoskeleton to provide both mechanical stability and transduction of extracellular signals into the cell (152). Adherens junctions are composed of cadherins bound together in a homotypic and $Ca^{2+}$-dependent fashion to link adjacent EC (81). Cadherins interact through their cytoplasmic tail with the catenin family of intracellular proteins, which in turn provide anchorage to the actin cytoskeleton (1). The primary adhesive protein present in human endothelial adherens junctions, vascular endothelial (VE) cadherin (29), is critical to maintenance of EC barrier integrity as demonstrated by increased vascular permeability induced in mice after infusion of VE-cadherin-blocking antibody (25). Similarly in cultured EC, VE-cadherin-blocking antibody increased permeability (124) and enhanced neutrophil transendothelial migration while producing reorganization of the actin cytoskeleton (72). The observation that VE-cadherin-blocking antibodies produce barrier disruption primarily in the alveolar capillary bed (25) suggests that differential adherens junction functioning exists within segments of the pulmonary vasculature. The MAPK pathway may be involved in regulating adherens junction/VE-cadherin function because MAPK inhibitors attenuate vascular endothelial growth factor (VEGF)-mediated VE-cadherin rearrangement and subsequent EC monolayer permeability (83).

Tyrosine phosphorylation may provide an additional regulatory link between actin cytoskeletal rearrangement and adherens junction function. Pervanadate treatment of cultured cells resulted in tyrosine hyperphosphorylation of catenins, partial dissociation of the catenin-cadherin complex, and subsequent decreased cell-cell adhesion (107). Similarly, the anti-adhesive protein thrombospondin-1-induced tyrosine phosphorylation of adherens junction proteins, actin rearrangement, and increased albumin flux across EC monolayers, whereas tyrosine kinase inhibition attenuated these effects (62). Recent work suggests that, in addition to increasing contractile forces, thrombin also alters EC permeability through dissociation of the tyrosine phosphatase, SHP2, from VE-cadherin complexes to produce increased tyrosine phosphorylation of catenins and subsequent destabilization of adherens junction linkage to the cytoskeleton (145). However, a certain level of basal tyrosine phosphorylation is likely necessary for maintenance of cell-cell contacts since selective inhibition of specific tyrosine kinases can disrupt these attachments (41).

A critical function of the EC barrier is the regulation of neutrophil (PMN) margination and migration into sites of acute inflammation, a complex process involving cytokine/chemokine signaling and interaction of specific recognition molecules (e.g., platelet endothelial cell adhesion molecule-1) on PMNs and ECs (154). As evidence for an integral role for the EC cytoskeleton and its connections in PMN diapedesis, disruption of either the EC actin cytoskeleton with cytochalasin B or stabilization of microtubules with paclitaxel decreases leukocyte transendothelial movement, whereas disas-

sembly of microtubules increases PMN migration (85). The ability of activated PMNs to increase EC permeability suggests that cross-cellular signaling pathways are employed during the cytoskeletal rearrangements of PMN transendothelial migration (57, 120). Binding of PMN to EC causes disruption of adherens junctions, as evidenced by the disappearance of VE cadherin and catenins from cell-cell contacts (30). Adherens junctions appear integral to this process because VE-cadherin-blocking antibodies increase PMN diapedesis (72), whereas conversely tight junctions remain intact during this migration (15). The signaling pathways involved in PMN diapedesis are not completely understood; however, elevation of intracellular $Ca^{2+}$ likely plays a role (73). Tyrosine phosphorylation pathways also appear important because activated PMNs increase the phosphotyrosine content of VE cadherin and β-catenin in association with adherens junctions disruption and hyperpermeability (142).

Tight junctions consist of transmembrane proteins such as occludin, the claudins, and junctional adhesion molecules coupled to cytoplasmic proteins, including the ZO family (104). Tight junction-associated cytoskeletal proteins such as ZO-1 appear to participate in signal transduction and to provide a link between occludin and the actin cytoskeleton (40). The functional significance of confocal microscopy-observed colocalization of F actin and ZO-1 is supported by the finding that cytochalasin D inhibits cytokine-induced fragmentation of ZO-1 interendothelial staining (13). Alterations in tight junctions may be signaled through the MAPK pathway, as both VEGF and $H_2O_2$-induced occludin dissociation from cell junctions and EC barrier dysfunction were partially blocked by MAPK inhibitors (82, 83).

Focal adhesions comprise extracellular matrix (ECM) proteins (collagen, fibronectin, laminin, vitronectin, proteoglycans), transmembrane integrin receptors, and cytoplasmic focal adhesion plaques (containing α-actinin, vinculin, paxillin, and talin), which combine to provide additional adhesive forces in barrier regulation and form a critical bridge for bidirectional signal transduction between the actin cytoskeleton and the cell-matrix interface (78, 128). ECM protein composition modulates basal EC permeability as well as TNF-induced barrier dysfunction (113, 158), whereas antibodies to $\beta_1$-integrin alter EC attachment, cell spreading, and permeability (92). Extracellular stimuli can be transmitted to the cytoskeleton through focal adhesion rearrangement linked to integrin ligation. Unliganded integrins are not associated with the cytoskeleton; however, ECM binding induces the attachment of integrins to intracellular actin fibers (42), a process in EC that stimulates tyrosine phosphorylation of multiple proteins [including paxillin, cortactin, and focal adhesion kinase (FAK)] as well as tyrosine phosphorylation-dependent $Ca^{2+}$ influx (10, 11). Integrin binding also targets activated extracellular signal-regulated kinase to newly formed focal adhesion sites (44). Reciprocally, intracellular signaling pathways that regulate cytoskeletal rearrangement can also modulate cell-matrix contacts. Rho inhibition dissociates stress fibers from focal adhesions, decreases phosphotyrosine content of paxillin and FAK, and enhances EC barrier function (18). Similarly, v-Src-induced tyrosine phosphorylation of focal adhesion proteins is a well-established stimulus for disassembly of these adhesive structures (43); however, some basal level of tyrosine phosphorylation appears necessary to maintain focal adhesions because selective tyrosine kinase inhibition will disrupt these contacts (41). In support of a barrier maintenance function for tyrosine phosphorylation of focal adhesions is a recent report describing the association of diperoxovanadate-induced transient EC barrier enhancement with phosphotyrosine incorporation into FAK (54). Further studies to clarify the role of barrier-protective and barrier-disruptive tyrosine kinases in both focal adhesion and adherens junction complexes will increase our understanding of EC barrier regulation by tethering forces.

## PULMONARY VASCULAR CYTOSKELETAL AND BARRIER REGULATION BY MECHANICAL FORCES

The study of EC in static culture provides the opportunity to mechanistically evaluate the role of cytoskeletal components in physiological functions. However, it has been increasingly appreciated that this approach may have major limitations given that the pulmonary endothelium in its native state is continuously exposed to mechanical forces that greatly influence cellular structure and function. Shear stress activates signaling pathways (e.g., MAPK), leading to upregulation of transcription factors and subsequent gene expression of various vasoactive substances, growth factors, and adhesion molecules (22, 60). Active cytoskeletal rearrangement begins rapidly and continues to occur over several hours as ECs orient themselves to reduce both peak shear stresses and shear stress gradients (5, 47). The cellular mechanisms for sensing flow and transducing its signal are still unclear, but recent reports suggest that both apical actin stress fibers linked to cell-cell contact sites and integrin-mediated signal transduction are involved (20, 79).

When EC in static culture are exposed to shear stress, multiple signaling pathways implicated in cytoskeletal rearrangement are stimulated, including $Ca^{2+}$ mobilization, G-protein activation, increased tyrosine phosphorylation, and MLCK and MAPK activation (65, 77, 130). These pathways interact downstream to produce the complex cellular effects of flow. For example, during shear stress, the GTPases Rho and Cdc42 combine to activate MAPKs; however, individually, Rho is necessary for flow-induced stress fiber formation and cell alignment and Cdc42 activates transcription factors (95). The integrated effects of these shear-induced signals on EC barrier function are variable depending on the magnitude, duration, and gradient of flow. Shear stress maximally increases protein expression of integrins after 12 h of exposure and significantly enhances cell-matrix attachment, suggesting that flow helps maintain the EC monolayer

through augmentation of focal adhesions (146). However, ECs exposed to high shear gradients, or turbulent flow, develop increased permeability relative to areas of either constant laminar flow or no flow (116). The majority of these studies have been performed using systemic circulation EC, and pulmonary EC-specific responses to flow are not well understood. One mechanism by which shear stress may alter barrier function is by inhibition of EC apoptosis (31); however, our recent work using a TNF model under static conditions suggests distinct signaling and cytoskeletal involvement in cytokine-induced apoptosis and permeability (115).

Ventilator-induced lung injury, a topic recently reviewed in this journal (32), is a highly morbid clinical entity believed to be caused by excessive mechanical stretch of pulmonary airways and vasculature, producing fluid flux across capillaries primarily through an active endothelial response (33, 109, 110). Similarly, the contribution of capillary rupture in this process has recently been reviewed in this journal (157). Intracellular $Ca^{2+}$ and tyrosine phosphorylation-dependent pathways appear to mediate the response to cell stretch (111, 112). In addition, reduction in endothelial tensile forces by MLCK inhibition significantly attenuates capillary leak in this model, illustrating the importance of the EC contractile apparatus in stretch-induced pulmonary edema (108).

The mechanism by which mechanical signals are transduced to the EC cytoskeleton is unclear but may involve the complex array of proteins that constitute the EC glycocalyx. The glycocalyx is a meshwork of glycoproteins and glycolipids that combine to form a cell-surface layer of anionic polymers that is variable across the vasculature (67). The components of the glycocalyx have been implicated in cytoskeletal organization as both syndecan, the primary heparin sulfate proteoglycan, and podocalyxin, the primary sialoprotein, modulating cell-cell and cell-matrix adhesion through their cytoplasmic domains (27, 35, 136). NMR techniques have demonstrated that cell-surface proteoglycans behave as viscoelastic anionic polymers, undergoing shear-dependent conformational changes, which may function as blood-flow sensors to transduce signals into the EC (132, 133).

## FUTURE DIRECTIONS

Although significant progress has been made in understanding the cellular and molecular events that regulate permeability, the goal of modulating the EC barrier in a clinically advantageous way remains elusive. Mechanistic investigation of known barrier-enhancing agents is essential to the development of novel therapeutic approaches, as evidenced by the failure of various clinical trials attempting to reduce the effects of individual cytokines or other mediators during sepsis or acute respiratory distress syndrome. One exciting exception is the recent report of significant mortality reduction in septic patients treated with recombinant activated protein C (8), although the exact mechanism by which activated protein C reduces microvascular injury is poorly understood. An alternative strategy to protect barrier integrity might instead focus on the downstream EC cytoskeletal rearrangements critical to the development of leakiness or barrier restoration. For example, agents designed to prevent the EC cellular contraction via reductions in hyperphosphorylation known to be essential for several models of agonist-induced pulmonary edema and ventilator-induced lung injury might facilitate restoration after barrier dysfunction occurs. It is therefore important to identify key regulatory events and effectors responsible for cytoskeletal modulation of pulmonary vascular permeability to hopefully provide a basis for the development of effective therapeutic interventions targeted toward the cytoskeleton. Further understanding of barrier restorative or barrier preserving mechanisms likely involved after short-acting edemagenic agents may provide valuable clues as to which therapies may shorten the edemagenic phase.

The study of angiogenesis may provide encouraging insights into mechanisms regulating EC permeability, a key event in angiogenic processes. For example, transgenic mice overexpressing angiopoietin-1 develop blood vessels resistant to the barrier-disruptive effects of inflammatory agents (140), whereas acute adenoviral vector delivery of angiopoietin-1 blocked VEGF-induced EC permeability (139). Recent in vitro work suggests angiopoietin-1 exerts its barrier protective effects by strengthening cell-cell contacts (48). Long recognized as critical to the integrity of the microvasculature (59), the barrier maintenance properties of platelets and platelet-derived phospholipids such as lysophosphatidic acid (37) and sphingosine 1-phosphate (J. G. N. Garcia, F. Liu, A. D. Verin, A. Birukova, M. A. Dechert, W. T. Gerthoffer, and D. English, unpublished observations), a distinct angiogenic factor, provide additional targets for exploration of pathways that enhance vascular integrity.

Finally, a viable area of interest concerns the physiological diversity of ECs along pulmonary segments, and, although at present the role of the cytoskeleton in the differential barrier properties of macrovascular vs. microvascular EC is unknown, exploration of this question may provide valuable insights into the regulation of pulmonary EC barrier function. The burgeoning field of DNA microarray technology provides an extremely powerful tool for this type of phenotypic analysis. With the Human Genome Project producing a wealth of sequence data, expression profiles of thousands of genes can now be assayed quickly and simultaneously (34, 68). Identification and analysis of single nucleotide polymorphisms in candidate cytoskeletal genes with defined barrier regulatory properties may also take us closer to understanding issues of individual variability in disease severity and therapy responses in edemagenic states. These exciting techniques provide an opportunity to determine critical regulatory genes responsible for complex processes such as barrier regulation and may reveal multiple novel targets for therapeutic intervention.

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